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Interaction of melittin with glycosphingolipids and phospholipids in mixed monolayers at different temperatures. Effect of the lipid physical state

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The influence of the liquid-expanded or liquid-condensed state of the lipid interface induced by changes of temperature on the lipid-protein interactions and their two-dimensional miscibility was studied for mixtures of melittin with different phospholipids (DPPC, DMPC, DOPC egg PC) and gangliosides (G_{M1}, G_{D1a}) in mixed monolayers at the air/145 mM NaCl interface. The critical amount of melittin at which a phase separation takes place in the mixed film increases as the glycosphingolipid or phospholipid is more liquid-expanded. The lipid-protein interaction increases the stability of both melittin and the lipid. The interaction of melittin with gangliosides is thermodynamically more favorable as these are more liquid-expanded. The interaction of melittin with phospholipids, on the other hand, is more favorable when the lipids are in the liquid-condensed state even if these films show lateral immiscibility at a lower proportion of protein compared to lipids in the liquid-expanded state. Hydration-dehydration effects in the polar head group region are likely to participate in these lipid-protein interactions.

Introduction

The surface properties and the temperature dependence of the physical state of phospholipids in monolayers has been well documented [1,2]. The thermotropic behavior of glycosphingolipids in monolayers has been recently described [3] and shown to correlate well with their phase transition properties in dilute aqueous dispersions [4]. In previous studies we investigated the behaviour of lipid protein mixed monolayers spread directly at the air/145 mM NaCl interface and the penetration of different proteins into pre-formed monolayers of neutral and anionic glycosphingolipids, DPPC, PC and phosphatidic acid. The major conclusions from these studies indicated that the individual surface properties of both the lipid and protein components are modified as a consequence of lipid-protein interactions and that the amount of area covered by the protein in the mixed film is an important parameter for establishing lateral phase separation [5,6]. It was also shown that it is possible to obtain homogeneously mixed protein-containing interfaces with a high proportion of protein and a stability that is indistinguishable from that corresponding to monolayers of pure lipids.

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Abbreviations: Cer, ceramide (*N*-acylsphingosine); NeuAc, *N*-acetylneuraminate; G_{M1}, Galβ1-3GalNAcβ1-4Gal(3-2α-NeuAc)β1-4Glc-1-1Cer; G_{D1a}, NeuAcα2-3Galβ1-3GalNAc-β1-4Gal(3-2αNeuAc)β1-4Glc-1-1Cer; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimiristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine, PC, egg yolk phosphatidylcholine.

Employing monolayers of glycosphingolipids or phospholipids that exhibit a different physical state at a single temperature it was concluded that the phase state of the lipid interface influences the two-dimensional miscibility and interactions with proteins [5,6]. However, several factors related to the properties of the polar head group and hydrocarbon moiety that vary for different glycosphingolipids [7–10] and phospholipids [1] are difficult to separate from the effect of the liquid-expanded or liquid-condensed character itself on the lipid-protein interactions. To explore this influence in more detail we have now studied the thermodynamics of mixing and the interactions of mixed monolayers of melittin with DMPC, DPPC, DOPC, egg PC and gangliosides G_{M1} and G_{D1a} at different temperatures for each system.

Materials and Methods

Synthetic phospholipids were from Sigma Chem. Co. (St. Louis, MO, U.S.A.) Egg PC and glycosphingolipids were purified and stored as described previously [5,11]. The technique, equipment, calculations and reproducibility for the measurement of monolayer properties at the air/145 mM NaCl interface at different temperatures was described in detail elsewhere [3,6,7]. Films of melittin with phospholipids were spread from chloroform/methanol (2 : 1, v/v) solutions, for gangliosides the spreading was performed from chloroform/ methanol/ water (40 : 20 : 3, v/v) solutions. The lipid-protein films were stable and reproducible under successive compression and expansion cycles indicating that no desorption of protein or monolayer solubility takes place during compression. The properties of the films were independent on the compression rate. This was checked in the range of $3.6 \cdot 10^{-4}$ to $7.1 \cdot 10^{-4}$ $m^2 \cdot min^{-1}$; depending on the amount of film spread in each case this results between $1.8 \cdot 10^4$ to $1.4 \cdot 10^5$ $m^2 \cdot mol^{-1} \cdot min^{-1}$ for the different monolayers studied.

Results and Discussion

Surface properties of melittin at different temperatures

In a previous paper we described the molecular behaviour, stability and the possibility of spreading and adsorption of pure protein films of representative membrane and soluble proteins at the air/water interface [6]. The conclusions derived from this report indicate that the maximal surface pressure acquired by a pure protein film falls between 12 and 25 $mN \cdot m^{-1}$ and no simple distinction is apparent between some proteins considered as soluble, extrinsic or integral of membranes.

The isotherms of melittin at different temperatures (5–40°C) are similar to that previously obtained at 20°C [5,6]. Fig. 1 shows the collapse pressure and the limiting molecular area of melittin at two different pH values. Both parameters are affected less than 5% by the changes in temperature. On alkaline subphases, a higher collapse pressure, lower limiting molecular area (fig. 1) and decreased surface potential per molecule (not shown) are found. These changes appear to be due to deprotonation of the basic amino acids located at the hydrophilic end and are in agreement with the assumption of a molecular conformation for the amphipathic peptide oriented as an α-helix

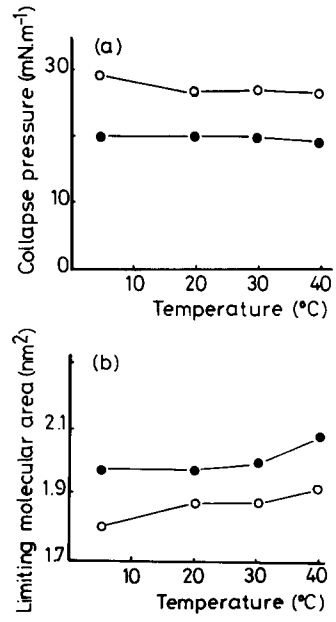

Fig. 1. Collapse pressure and limiting molecular area of melittin at different temperatures and pH. Collapse pressure (a) and limiting molecular area (b) on subphase of 145 mM NaCl at pH 5.6 (●) or 10.6 (○).

perpendicular to the interface [6]. The surface behaviour on subphases at pH 1.2 (not shown) is very similar to that obtained at pH 5.6.

The essentially unmodified surface behaviour of melittin at different temperatures allows a simpler analysis for mixtures in which the surface parameters of the lipids are influenced by temperature. Besides, these results indicate that the free energy of compression of melittin does not change by temperature and the entropy of compression is therefore null. In other words, an unfavorable decrease in configurational entropy at the interface caused by an increase of the interfacial ordering of protein molecules as these are compressed from $\pi \rightarrow 0$ up to a closely packed array can probably be counterbalanced by a favorable increase of the system's entropy if water molecules are released from the interfacial region. This is similar to what was suggested for fully liquid-expanded monolayers of glycosphingolipids [3].

Mixed lipid-protein films

(i) Stability and lateral phase separation. Lateral immiscibility in monolayers can be established in mixed glycosphingolipid protein films when the protein concentration at the surface exceeds some critical value. The critical amount of melittin at which the lateral phase separation takes place increases as the glycosphingolipids are more liquid-expanded [6]. When these mixed ganglioside-melittin films are run on a subphase at pH 1.2, in conditions of protonated sialosyl residues and a more liquid-condensed state [7], or at lower temperature (5°C), the maximal molar fraction of the protein accepted in the film before becoming inmiscible decreases but the effect of the complexity of the polar head group is still present (Table I). The individual surface behaviour of both melittin (see above) and the more complex gangliosides at 5°C were similar to those obtained at 20°C [3]. These results indicate that, apart from the amount of protein and the physical state of the lipid, the temperature factor itself has an important effect in the demixing process of lipid-protein mixtures. At high protein molar fractions the lipid-protein films appear as homogeneous and exhibit a single collapse pressure because at protein area contributions above 90% the behaviour of the mixed interface is similar to that of pure protein and any influence of the lipid results undetectable (Fig. 2a).

TABLE I

CRITICAL MOLAR FRACTION OF MELITTIN ALLOWING FOR A MONOPHASIC SYSTEM WITH GANGLIOSIDES

The value of molar fraction indicates the surface protein concentration at which an immiscible behaviour becomes evident and the mixed lipid-protein interface undergoes lateral phase separation. Below this protein molar fraction the mixed monolayer has a homogeneous behaviour (see text). The percentage of protein area contribution shown in brackets is calculated from the molecular area and molar fraction of the protein in the mixed films.

Lipid	Molar fraction of melittin		
	Subphase pH 5.6		Subphase pH 1.2
	20°C	5°C	20°C
G_{M1}	0.5 (75)	0.33 (63)	0.25 (55)
G_{D1a}	0.75 (87)	0.50 (71)	0.33 (57)

The pattern for mixtures of melittin with phospholipids is similar to that found for systems containing glycosphingolipids. Fig. 2b shows the collapse pressure of films of DMPC-melittin at several molar fractions of protein. At molar fractions below 0.05 a mixed monophasic system is obtained with a single collapse pressure that is higher compared with the value of pure DMPC. When the composition of the monolayer approaches the critical value of protein concentration that induces phase separation, a lower collapse point than that exhibited by the monophasic system gradually shows up. This lower collapse point is 10–15 $mN \cdot m^{-1}$ above that of pure melittin, probably indicating the formation of lipid-protein associations or a greater stability acquired by the protein in presence of lipid [5]. As the amount of protein in the mixed system increases, the surface stability of the lower collapsing phase decreases and the value of the collapse pressure approaches that of the pure protein. The higher collapse pressures in the biphasic region are always 6–7 $mN \cdot m^{-1}$ above that of the pure phospholipids indicating that the lipid-protein interaction alters the stability of the rest of the lipid phase; this effect was previously found for the compression of protein-penetrated films of glyco-

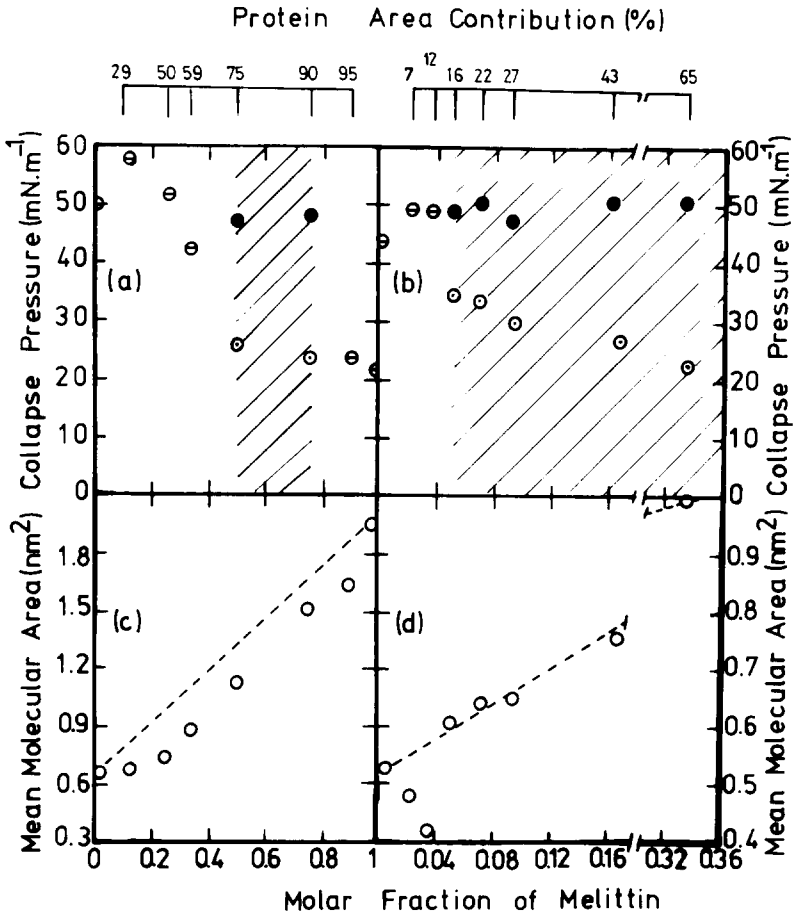

Fig. 2. Collapse pressure (upper part) and limiting molecular area (lower part) of mixed monolayers of melittin in different molar fractions with G_{M1} (a and c) or with DMPC (b and d). The single (⊖), lower (⊙) and higher (●) collapse pressure points and the region of lateral immiscibility (stripped region) are indicated for different molar fractions of melittin. The subphase was 145 mM NaCl at pH 5.6 and 20°C. The protein area contribution was calculated as indicated in Table I. The straight dashed lines in (c) and (d) correspond to the variation of the molecular area with the composition according to the additivity rule for an ideally mixed film.

sphingolipids [5]. This greater stability may also be due to some lipid-protein association that does not mix with the rest of the protein-rich phase.

The results obtained in monolayers can be compared with those carried out in bulk systems. Using Raman spectroscopy, Verma and Wallach [12] have reported that for DMPC-melittin systems (28 : 1 and 56 : 1, molar ratio) the transition temperature of the mixture is increased by 11 and 5 K, respectively, compared to that of pure DMPC. These authors suggested that this effect may be explained if melittin imposes a long range organization in the bilayer through non-polar interactions. This idea is in agreement with the monolayer behaviour since the mixed interface is homogeneous and exhibits a higher stability than pure DMPC at comparable lipid-protein ratios. Using the same technique, Lavialle et al. [13] described two phase transition temperatures (14–17°C and 29–30°C) for mixtures of DMPC and melittin at lower lipid-protein ratios (14 : 1 and 10 : 1). The lower transition point was associated to a depression of the main transition temperature compared with pure DMPC (23°C) and the higher transition was associated to a possible phase change of the lipid immobilized at the lateral lipid/protein in-

terface. According to these authors, at 20°C two kinds of lipid behaviour would coexist with different thermotropic behaviour induced by the protein molecules. This interpretation is also in agreement with our results. At comparable molar ratios to those employed in the above calorimetric studies, the mixed monolayer undergoes lateral phase separation (see above and Fig. 2) with a lower collapse point that is higher than that of pure protein films and a second collapse point higher than that of the pure lipid. Indeed, the monolayer system does not allow conclusions on whether a putative lipid-protein complex collapsing at the lower pressure point corresponds to the lower or higher melting component observed by Lavialle et al. [13]. However, the monolayer data allow to clearly ascertain the presence and coexistence of two phases in the mixed lipid-protein interface at high surface pressure, with a behaviour that is different to that of the pure components.

The influence of the physical state of the phospholipids on the lipid-protein behaviour was studied with phosphatidylcholines containing different hydrophobic chains. The general results are similar to those found for glycosphingolipids (Fig. 3). Namely, the amount of protein at the interface leading to lateral phase separation increases as the phospholipids are more liquid-expanded. For a fixed DPPC/melittin ratio (12:1), the mixed monolayer at lower temperature (see Fig. 4 at 20°C and 28°C) shows two-dimensional immiscibility. When the temperature is set to 32°C a new component is detected with a higher stability to collapse than the pure protein and immiscible with the rest of the lipid phase. The mixture becomes completely homogeneous with a single collapse point at a temperature in which pure DPPC shows a fully liquid-expanded behaviour (see inset in Fig. 4). These results are giving experimental support to the lipid-protein model proposed by Loockman et al. [14] in which, using computer simulation studies, large stable protein clusters and separation of a protein-rich phase are

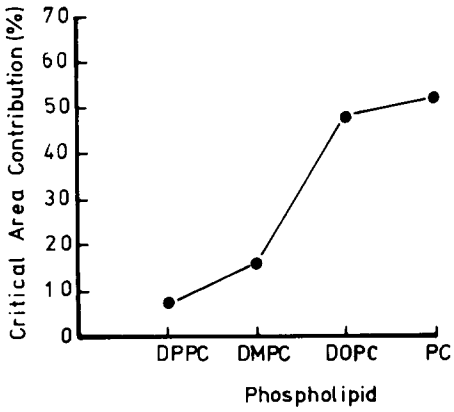

Fig. 3. The critical area contribution (%) in the ordinate corresponds to the maximal percentage of melittin area contribution allowing for a monophasic system for mixed monolayers with the phospholipids indicated. The subphase and calculation of the protein area contribution is as indicated in Fig. 2. PC corresponds to egg PC.

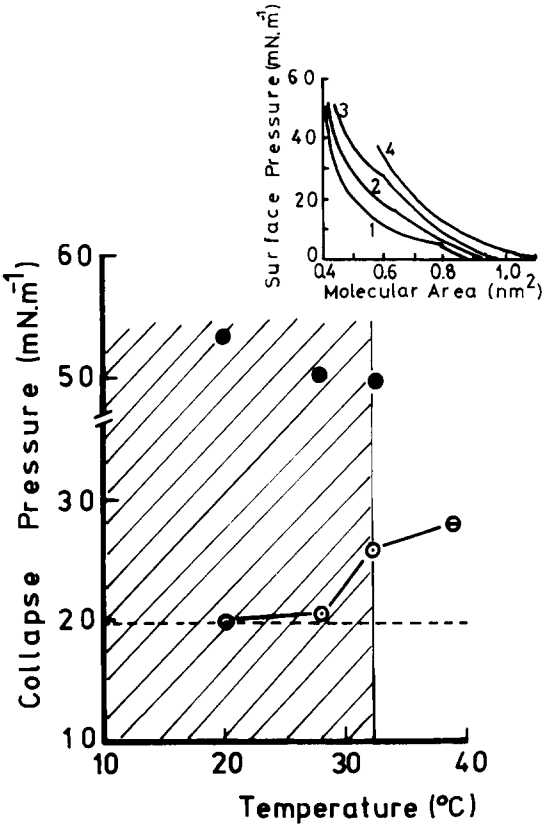

Fig. 4. Effect of temperature on the miscibility of mixed monolayers of melittin with DPPC. The lower (⊙), higher (●) or single (⊖) collapse points are shown for a mixed monolayer of DPPC-melittin at a molar ratio 12:1 at different temperatures. The dashed line indicates the collapse pressure of a monolayer of pure melittin. The stripped region corresponds to biphasic systems. Inset: isotherms for pure DPPC at 20°C (1); 28°C (2); 32°C (3) and 40°C (4).

more likely to occur as the temperature of the system decreases and passes below the transition temperature of the phospholipid.

(ii) Excess free energy of mixing. The excess free energy of mixing (ΔG_{xs}) can be calculated from the difference between the integrated areas under the pressure-area isotherm of the experimental and ideal films up to a specified surface pressure (cf. Refs. 6 and 15). The effect of temperature on ΔG_{xs} of the lipid-protein interfaces studied is shown in Fig. 5. In general ΔG_{xs} becomes more negative as the temperature of the system increases indicating a thermodynamically more favored mixing. At a particular temperature the excess free energy of mixing is more negative for glycosphingolipids with a lower bulk transition temperature (cf. Ref. 4). On the contrary, the ΔG_{xs} for mixtures with phospholipids becomes negative as the respective bulk transition temperature is higher (Fig. 5). At 40°C the isotherms of both DOPC and DPPC are liquid-expanded and, at the molar proportion indicated in Fig. 5, the protein area contribution is similar (about 23%); however, the ΔG_{xs} is more negative in the case of DPPC suggesting that the interaction of melittin with it is more favored than with DOPC. The behaviour at different temperatures of lipids with a similar physical state can be analysed for the case of DMPC at 20°C compared to DPPC at 40°C. At each of these temperatures, DMPC and DPPC are very near their bulk transition temperature and have a similarly liquid-expanded character. However, the critical point of protein proportion in the mixed film for obtaining an immiscible behaviour is 2.5-times greater for DPPC/melittin mixtures at 40°C (not shown) than for DMPC/melittin at 20°C (fig. 2). Similar to the case of glycosphingolipids (see Table I and above) these results indicate that the temperature factor has an additional effect on the mixing of phospholipids with proteins that leads to a progressive increment of the lateral miscibility.

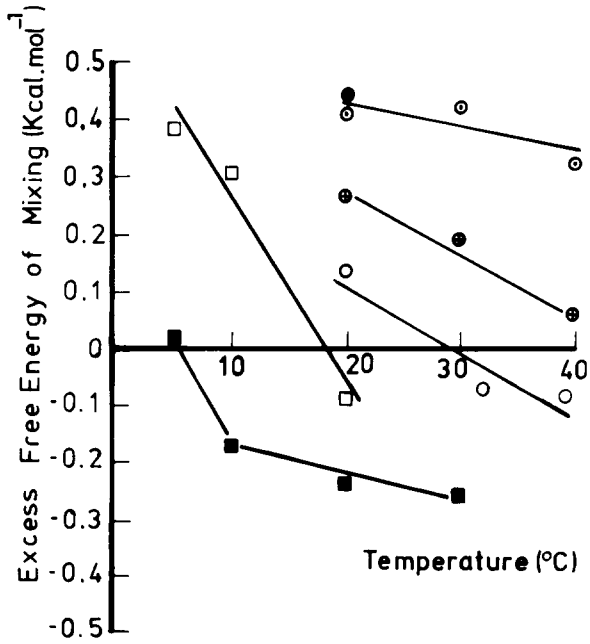

Fig. 5. Excess free energy of mixing at different temperatures for mixed monolayers of melittin with glycosphingolipids and phospholipids at the molar ratios indicated in brackets: G_{D1a}-melittin (3:1) (■); G_{M1}-melittin (3:1) (□); DPPC-melittin (12:1) (○); DMPC-melittin (28:1) (⊕); DOPC-melittin (10:1) (⊙); egg PC-melittin (7:1) (●).

The pattern that emerges from these results indicates that the interaction of phospholipids with melittin is thermodynamically more favorable when the lipid is in the liquid-condensed state compared to the liquid-expanded but the percentage of protein area contribution accepted in the system before undergoing lateral phase separation is lower. According to the results, the more liquid-expanded phospholipids would accept a greater area of protein and exhibit a greater lateral stability even if the intermolecular interactions are less favored; all this leads to a further increment of the liquid-expanded state and molecular delocalization of the mixed interface.

In principle, from the slope of the curves in Fig. 5 it is possible to estimate the excess entropy of mixing (ΔS_{xs}). The sign of this thermodynamic parameter results positive and the average values fall between 4 e.u. and 35 e.u. depending on the type of lipid and the range of temperature considered for the estimation. The relatively high positive values for ΔS_{xs} compared to the ideal values for the entropy of mixing (approx. 1 e.u. at 3:1 lipid/protein molar ratio assuming molecules of similar size), suggest that values of excess enthalpy of mixing (ΔH_{xs}) can be as high as 9 kcal/mol. With respect to the molecular interpretation of these values, Gaines [15] has raised the question

on how much of the ΔG_{xs} (and, by extension, ΔS_{xs} and ΔH_{xs}) is due to interactions between the film-forming molecules and how much results from interactions between the mixed molecules and the aqueous phase with respect to an ideal non-interacting behaviour. If these concepts are taken into account a probable explanation for the behaviour of the mixed films with melittin may be suggested. Although interactions of lipids with melittin occur with intermolecular condensation (especially noted for the highly liquid expanded gangliosides, see also Ref. 6) and with a higher stability with respect to the ideal behaviour, this takes place with a considerable entropy increase suggesting an increment of the interfacial disorder and with unfavorably high enthalpy. In the case of gangliosides this may be ascribed to some dehydration of their highly hydrated bulky polar head group [16] as a consequence of the lipid-protein interaction since the release of water molecules from the interface would lead to enthalpically unfavored and entropically favored changes. Supporting these findings, it has been recently reported that the interfacial microenvironment of liposomes containing phospholipids mixed with different gangliosides appears as more polar and this is reverted to a less polar environment by the presence of melittin [17]. In this analysis it cannot be ruled out that the protein conformation and the water molecules associated to the mixed interface may be contributing direclty to a more disordered state compared with an ideal mixture.

For the more complex gangliosides that can form micelles in bulk solution [4,18] the lipid-protein interaction is more favored in spite of the transition temperature of the glycosphingolipids being greater compared to DOPC or egg PC [4]. The interaction of basic proteins with gangliosides occurs with a thermodynamically favored reduction of the intermolecular spacings and with an increment of the stability to collapse with respect to the individual behaviour (Fig. 2). The increase of the molecular packing can be ascribed to a reduction of the molecular area of these complex glycosphingolipids or to a molecular cavity effect and it is not simply related to electrostatic interactions [6]. At pH 1.2 where the sialosyl residues are protonated [7] the reductions of molecular area induced by the presence of melittin in the mixed film is similar to that found with negatively charged gangliosides at pH 5.6 or 10.4.

The properties, behaviour and organization of lipid/protein interfaces can not be explained only on the basis of the surface properties of the individual components and the data must be considered as resulting from multivariable interdependent events in a complex system. These include variations of the physical state of the interface, the type, amount and state of the lipid, the fraction of total area contributed by the protein, the type of interactions and overall intermolecular organization and the structural state of water associated to the interface. All these are factors dynamically related that influence concomitantly short- and long-range effects that participate in lipid-protein interactions.

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